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Transcription-Coupled Methylation of Histone H3 at Lysine 36 Regulates Dosage Compensation by Enhancing Recruitment of the MSL Complex in *Drosophila melanogaster*▽

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In *Drosophila melanogaster*, dosage compensation relies on the targeting of the male-specific lethal (MSL) complex to hundreds of sites along the male X chromosome. Transcription-coupled methylation of histone H3 lysine 36 is enriched toward the 3′ end of active genes, similar to the MSL proteins. Here, we have studied the link between histone H3 methylation and MSL complex targeting using RNA interference and chromatin immunoprecipitation. We show that trimethylation of histone H3 at lysine 36 (H3K36me3) relies on the histone methyltransferase Hypb and is localized promoter distal at dosage-compensated genes, similar to active genes on autosomes. However, H3K36me3 has an X-specific function, as reduction specifically decreases acetylation of histone H4 lysine 16 on the male X chromosome. This hypoacetylation is caused by compromised MSL binding and results in a failure to increase expression twofold. Thus, H3K36me3 marks the body of all active genes yet is utilized in a chromosome-specific manner to enhance histone acetylation at sites of dosage compensation.

Similar to sex determination in mammals, sex determination in flies involves an unequal distribution of sex chromosomes, with females carrying two X chromosomes compared to one in males. The resulting difference in gene dose requires compensating mechanisms that guarantee equal expression of X-linked genes in both sexes. In mammals, dosage compensation involves transcriptional silencing of one of the two female copies of the X chromosome. In flies, the opposite strategy is realized, as transcription on the single male X chromosome is increased twofold.

Genetic screens in flies identified five proteins and two noncoding RNAs that are essential for the process of dosage compensation and whose absence causes male-specific lethality (MSL). The proteins MSL1, MSL2, MSL3, MLE (maleless), and MOF (males absent on the first) form a ribonucleoprotein complex (the MSL complex), together with the two noncoding RNAs, *roX1* and *roX2* (RNA on the X), which specifically binds to hundreds of sites on the male X chromosome and promotes transcriptional upregulation (16, 18, 25). This transcriptional upregulation is believed to involve histone hyperacetylation of dosage-compensated genes by the histone H4 lysine 16-specific histone acetyltransferase MOF (10).

Recent studies suggested that MSL complex binding sites fall into two categories: high-affinity sites which are able to bind partial MSL complexes consisting of MSL1 and MSL2 (in mutant backgrounds of MSL3, MLE, and MOF) and low-affinity sites which require the full complement of the MSL complex (8, 12, 17). It has been postulated that the presence of high-affinity sites creates high local concentrations of MSL complex members on the X chromosome, which subsequently allows MSL complex binding to sites of lower affinity. Chromatin immunoprecipitation coupled with DNA microarrays (ChIP-chip) profiling experiments showed that the MSL complex localizes primarily to the 3′ end of dosage-compensated genes (2, 9). Indeed, DNA elements in the 3′ end of these target genes have been shown to be required for MSL binding, yet the ability to recruit the MSL complex strongly depends on their transcriptional activity (6, 14). This suggests that recognition of target DNA sequences with relatively low affinity for MSL proteins either is dependent on high chromatin accessibility or requires an additional, transcription-coupled signal.

Interestingly, trimethylation of histone H3 at lysine 36 (H3K36me3) is a histone modification that has been shown to be enriched specifically toward the 3′ end of active genes (3, 20, 22, 23). In *Saccharomyces cerevisiae*, H3K36me is bound by the chromodomain protein Eaf3 and recruits the Rpd3S HDAC complex to remove transcription-coupled hyperacetylation, which could otherwise unmask internal transcription start sites (5, 11, 13). MSL3 is a *Drosophila* homologue of yeast Eaf3, opening the possibility that it interacts with methylated H3 lysine 36 to recruit the MSL complex to the 3′ end of dosage-compensated genes.

In this study, we investigated the relationship between H3K36 methylation and MSL complex recruitment using RNA interference (RNAi) and ChIP in male *Drosophila* SL2 cells. We show that H3K36me3 is enriched promoter distal at dosage-compensated genes and relies on the histone methyltrans-

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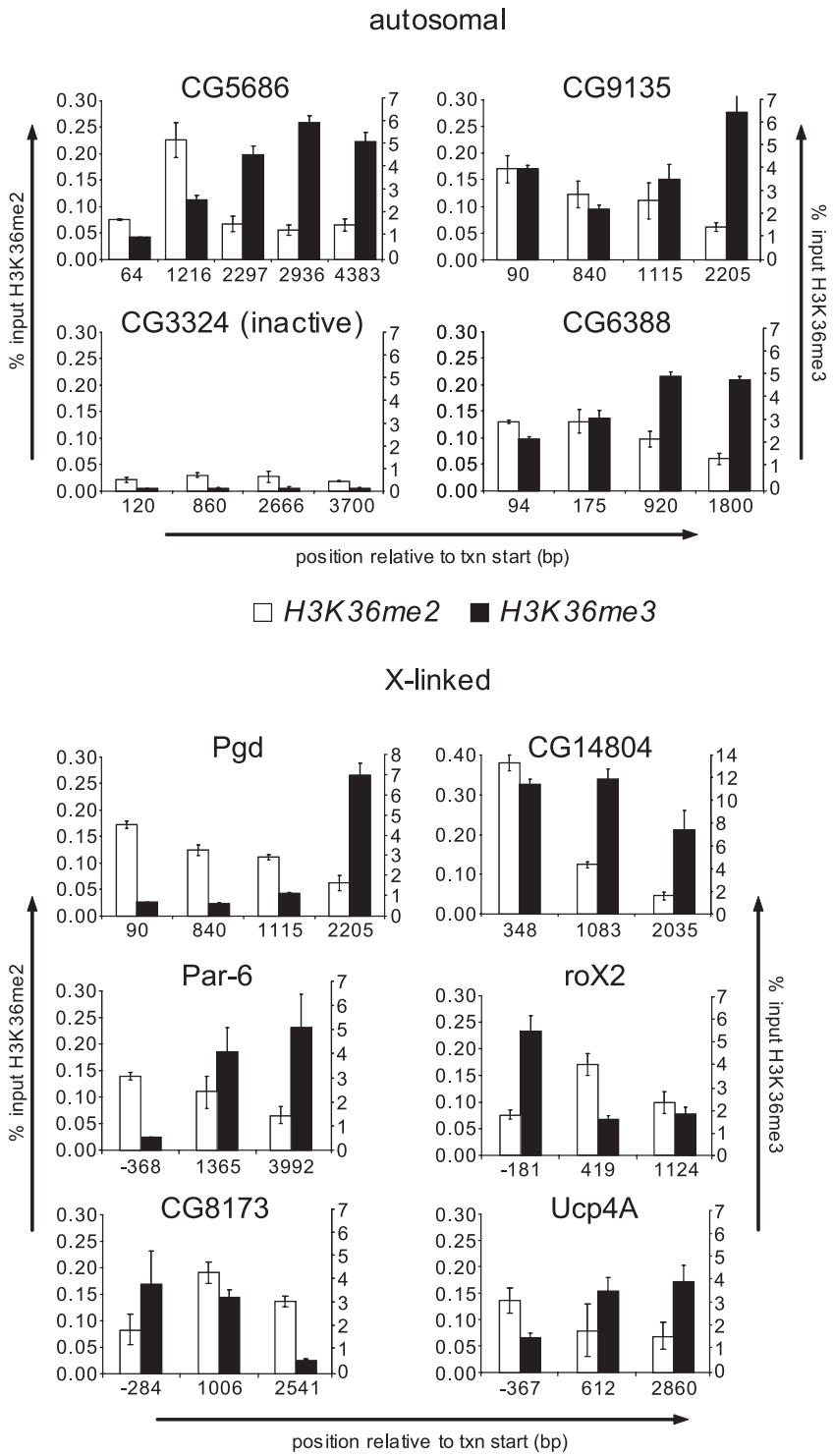


FIG. 1. High-resolution analysis of di- and trimethylation of H3K36 on autosomal and dosage-compensated genes. ChIP analysis of *Drosophila* SL2 cells using antibodies specific for H3K36me2 or H3K36me3 and quantification by real-time PCR. Shown are the average and standard deviation of ChIP enrichments from at least three independent experiments normalized to histone H3 occupancy. The *x* axis reflects the base pair position relative to the transcriptional start site. The *y* axis reflects enrichment (bound/input values are displayed as percent recovery of input DNA). H3K36me2, left scale; H3K36me3, right scale. Numbers in graphs are gene identification numbers according to Flybase.

ferase Hypb, similar to active autosomal genes (4). Despite comparable regulation, decreased H3K36me3 has an X-specific effect on the acetylation of H4 lysine 16 (H4K16ac), as it causes a reduction of that mark on dosage-compensated genes, while on autosomal genes, levels are increased. Hypoacetylation on the male X chromosome as a consequence of Hypb loss of function coincides with reduced binding of the MSL1 and MOF proteins. Importantly, compromised MSL recruitment

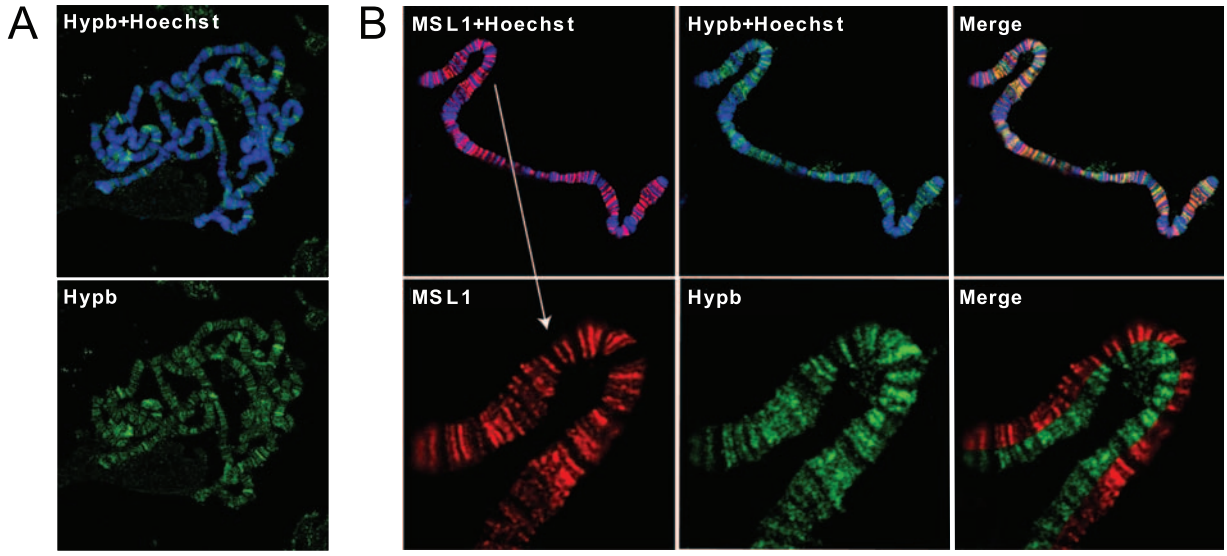


FIG. 2. Hypb binds autosomes and the male X chromosome. Polytene chromosomes of male third-instar larvae were stained with antibodies against Hypb (green) and MSL1 (red). DNA was visualized by Hoechst staining (blue). (A) Hypb localized preferentially to euchromatic interband regions, suggesting a general role in transcription. (B) Hypb partially localizes to sites of MSL1 enrichment along the X chromosome, consistent with a role in the trimethylation of H3K36 on dosage-compensated genes.

results in a failure to adequately upregulate the expression of a subset of X-linked genes. Thus, our data indicate that H3K36 trimethylation provides an important signal to attract MSL complex proteins to genes and further establish that the histone acetylation readout of H3K36 methylation in males is chromosome specific.

MATERIALS AND METHODS

Tissue culture of SL2 cells. *Drosophila* SL2 cells were kept in Schneider medium (Gibco) supplemented with 10% fetal calf serum.

RNAi in cultured SL2 cells. Double-stranded RNA (dsRNA) for RNAi knockdown of *Drosophila* Hypb (bp 3236 to 3944) was generated according to Ambion MEGAscript manual instructions. 1×10^6 SL2 cells were plated in 2 ml medium and treated with 70 μg dsRNA for 4 days. Treatment was repeated after cell splitting for a total of 7 days before harvesting cells for subsequent analysis.

Western blot analysis and antibodies. Western blottings were performed as previously described (4). Mouse monoclonal antibody against Hypb was used as previously described (4). Purified, bacterially expressed protein fragments were used to generate pMal-Hypb (amino acids [aa] 1 to 436), pMal-Hypb (aa 919 to 1135), and pMal-Hypb (aa 2040 to 2363), according to standard procedures. Hsp70 (mouse monoclonal; StressGen), H2A (Upstate 07-146), H3 (Abcam ab1791), H3K36me2 (Upstate 07-369), H3K36me3 (Abcam ab9050), H4K8ac (Upstate 07-328), H4K12ac (Upstate 07-595), H4K16ac (Upstate 07-329), MOF, and MSL1 (19) were used for the analysis.

ChIP. ChIPs of histone modifications, MOF and MSL1, were carried out as described previously (4).

Immunostaining of polytene chromosomes. Preparation of polytene chromosomes and immunostaining were performed as described previously (http://www.igh.cnrs.fr/equip/cavalli/Lab%20Protocols/Immunostaining.pdf). Hypb antibody and preserum were used in a 1:15 dilution; all other antibodies were used in a 1:250 dilution. Images were taken with a Leica Sp5 confocal microscope (Leica Microsystems, Mannheim) using an HCX PL APO 63.0× 1.40 oil objective.

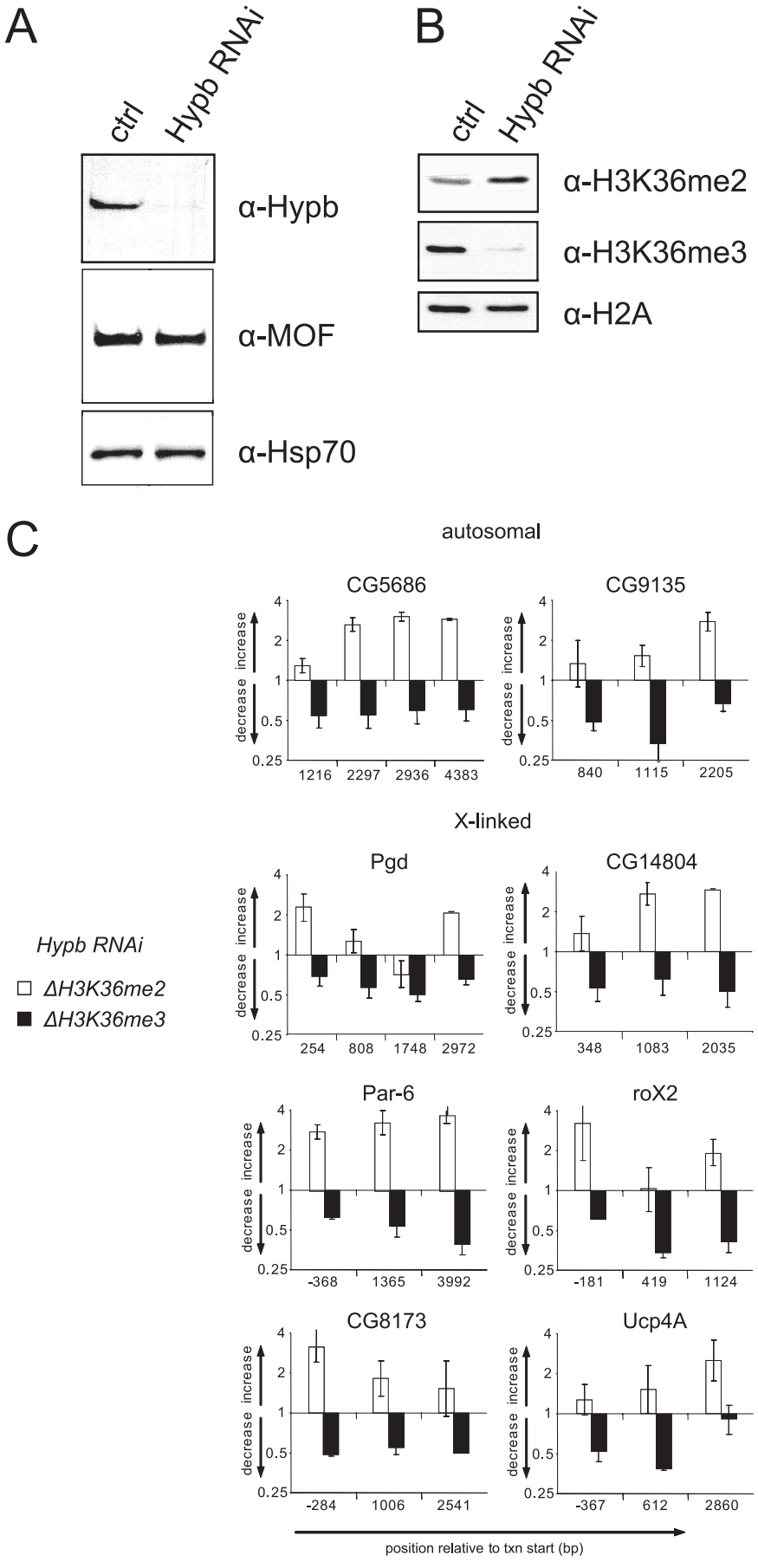
Reverse transcription and real-time PCR. Reverse transcription and quantitative real-time PCR analysis were performed as described previously (14). PCR conditions and autosomal primer sequences were as described previously (4, 28). Additional details for primer positions and sequences are available from the authors.

RESULTS

Distributions of H3 lysine 36 methylation states are similar at dosage-compensated and autosomal genes. To determine whether distribution of H3K36 methylation parallels the pattern of MSL binding, we performed ChIP with antisera specific for di- and trimethylation of this residue in male SL2 cells. ChIP enrichments were quantified by real-time PCR to determine K36 methylation states along selected X-linked and autosomal genes. On the X chromosome, both methylation states were enriched on a set of genes known to be subject to dosage compensation (14, 24, 26) yet displayed distinct patterns of localization. Dimethylation was preferentially localized proximal to promoters, whereas trimethylation peaked in the middle and in the 3′ ends of dosage-compensated genes (Fig. 1), reminiscent of MSL binding. To determine whether K36 methylation states are distinct at MSL target genes, we compared X-linked profiles with di- and trimethylation on autosomes. Both methylation states were enriched along the body of transcriptionally active autosomal genes and assumed localizations that generally mirrored those of dosage-compensated genes (Fig. 1). This is in agreement with the distributions that we obtained recently for individual autosomal genes as well as for chromosome-wide profiles in female Kc cells (4).

These results indicate that the H3K36 methylation signature at dosage-compensated genes on the male X chromosome is similar to that on autosomes.

Hypb mediates transcription-dependent trimethylation of H3 lysine 36 on autosomes and X chromosome. In *Drosophila*, trimethylation of H3K36 relies on the activity of the SET-domain-containing protein CG1716 (4, 15, 24a, 29). Based on homology to the human histone methyltransferase HYPB (27), we refer to CG1716 as Hypb. In female cells, Hypb is enriched on actively transcribed genes and coincides with H3K36 tri-



methylation downstream of promoters (4). We determined the chromosomal distributions of this enzyme in *Drosophila* males by immunofluorescence staining of polytene chromosome squashes isolated from third-instar larvae. At low resolution, Hypb displayed binding to interbands and puffs with no apparent preference for any particular chromosome (Fig. 2A). When we performed costaining with an antibody against MSL1, we observed partial colocalization at many sites along the X chromosome (Fig. 2B). Despite an extensive overlap with MSL1 binding, we did not detect a characteristic pattern of Hypb localization on the compensated X chromosome, suggesting that Hypb is present at all sites of active transcription.

To define the contribution of Hypb to H3K36 trimethylation, we reduced transcript levels by RNAi in male SL2 cells. Hypb knockdown reduced protein levels, as indicated by Western blot analysis (Fig. 3A), and resulted in a specific decrease in H3K36 trimethylation, while dimethylation was slightly increased (Fig. 3B). These bulk methylation changes are similar to the ones previously observed following RNAi knockdown in female Kc cells (4).

Next, we examined by ChIP if the bulk effects on H3K36 methylation recapitulate changes at individual loci on autosomes and the X chromosome. At two autosomal genes, knockdown of Hypb reduced the presence of trimethylation and coincided with an increase of H3K36 dimethylation (Fig. 3C). This reflected the RNAi effects on bulk methylation and was similar to changes detected for individual genes in female Kc cells (4). Moreover, we show that the same chromatin changes also occur at six dosage-compensated genes following Hypb RNAi in male SL2 cells (Fig. 3C).

We conclude that Hypb mediates trimethylation of H3K36 on autosomes and the X chromosome in male *Drosophila* cells.

H3K36 trimethylation is required for hyperacetylation of H4K16 on the dosage-compensated X chromosome. Having established that H3K36me3 is equally regulated on all chromosomes, we asked if trimethylation mediates distinct downstream effects at autosomal and X-linked genes. In budding yeast, H3K36 methylation has been reported to regulate acetylation of histones H3 and H4 on transcribed open reading frames through recruitment of an HDAC-containing complex (5, 11, 13). Reduction of H3K36 trimethylation in female *Drosophila* cells also affected histone acetylation levels in transcribed regions, yet only for H4 lysine 16 acetylation (4). We therefore investigated if H4K16ac is subject to differential regulation in male cells. ChIP in male SL2 cells revealed that H4K16ac peaks at promoters of active autosomal genes and is less abundant along gene bodies (see the supplemental data at http://www.fmi.ch/groups/schubeler.d/web/data.html) (J. Kind J. M. Vaquerizas, and A. Akhtar, unpublished data), similar to female Kc cells. In contrast, we detected highly elevated levels of H4K16ac along the gene body of dosage-compensated genes, which is in agreement with previous reports (9, 24). On these genes, H4K16ac was especially abundant in the 3′ end, reminiscent of the localization of H3K36me3 (see the supplemental data at http://www.fmi.ch/groups/schubeler.d/web/data.html).

Western blot analysis of Hypb knockdown showed that a reduction of trimethylation coincided with decreased levels of bulk acetylation at H4K16 (Fig. 4A). This was different from female cells where global acetylation increases in response to Hypb knockdown (4). Interestingly, when tested at specific loci by ChIP, we observed that levels of acetylation increased at autosomal genes yet at the same time decreased at dosage-compensated genes (Fig. 4B). We reasoned that this X-specific decrease is likely to account for the global reduction, since dosage-compensated genes show very high levels of H4K16 hyperacetylation compared to autosomal genes (see the supplemental data at http://www.fmi.ch/groups/schubeler.d/web/data.html) (24).

We conclude that while the presence of H3K36me3 reduces H4K16ac on autosomes, similar to female Kc cells, it has an additional male-specific function in enhancing H4K16ac on the dosage-compensated X chromosome. One possibility is that it contributes to MSL recruitment, which has previously been shown to be required for H4K16 hyperacetylation by MOF.

H3K36 trimethylation enhances recruitment of MSL proteins at dosage-compensated genes. The effect on H4K16ac suggests a reduction in MOF levels at target sites, indicating a function of H3K36me3 in MSL complex recruitment. To address this question, we reduced Hypb-dependent trimethylation and examined the levels of MSL recruitment in the same set of X-linked genes. We determined the binding pattern of two selected MSL complex members by ChIP using specific antibodies directed against MSL1 and MOF. Both proteins were bound along gene bodies, with the strongest enrichment found in the 3′ ends, reminiscent of H3K36me3 (Fig. 5A) and in agreement with previous studies (2, 9). The addition of Hypb dsRNA and the subsequent reduction of H3K36me3 strongly diminished the presence of MOF at the four target genes assayed (Fig. 5A), which was consistent with the reduction in H4K16ac at these genes. Interestingly, while MSL1 abundance was also reduced at *Par-6*, CG8173, and *Ucp4A*, it remained mostly unaffected at sites of the *roX2* gene. The *roX2* gene presents 1 of 30 to 40 high-affinity sites, which contain sequence elements that are able to attract MSL1 to the X chromosome even in the absence of complete dosage compensation complexes (12) or transcription (14). The fact that MSL1 binding at the *roX2* locus is mostly insensitive to Hypb RNAi indicates that strong sequence affinity can mediate robust recruitment independent of H3K36me3. However, this MSL1 interaction appears to be insufficient to recruit a fully functional MSL complex, as reflected by reduced MOF

FIG. 3. RNAi knockdown of Hypb has similar effects at autosomal and X-linked loci. (A) Western blot analysis using antibody specific for Hypb reveals efficient reduction of Hypb in male *Drosophila* SL2 cells. Hsp70 and MOF remain unaffected by RNAi knockdown and serve as loading controls. (B) Reduction of Hypb results in a reduction of H3K36me3 and a coinciding increase of H3K36me2. H2A serves as a loading control. (C) Levels of H3K36 methylation states in RNAi and control cells were compared by ChIP followed by real-time PCR analysis. Shown is the ratio of H3K36me enrichments (change [*n*-fold], *y* axis) of RNAi over control cells relative to the position from the transcription start site (*x* axis). Effects on H3K36 methylation states at individual loci reflect bulk changes upon Hypb knockdown.

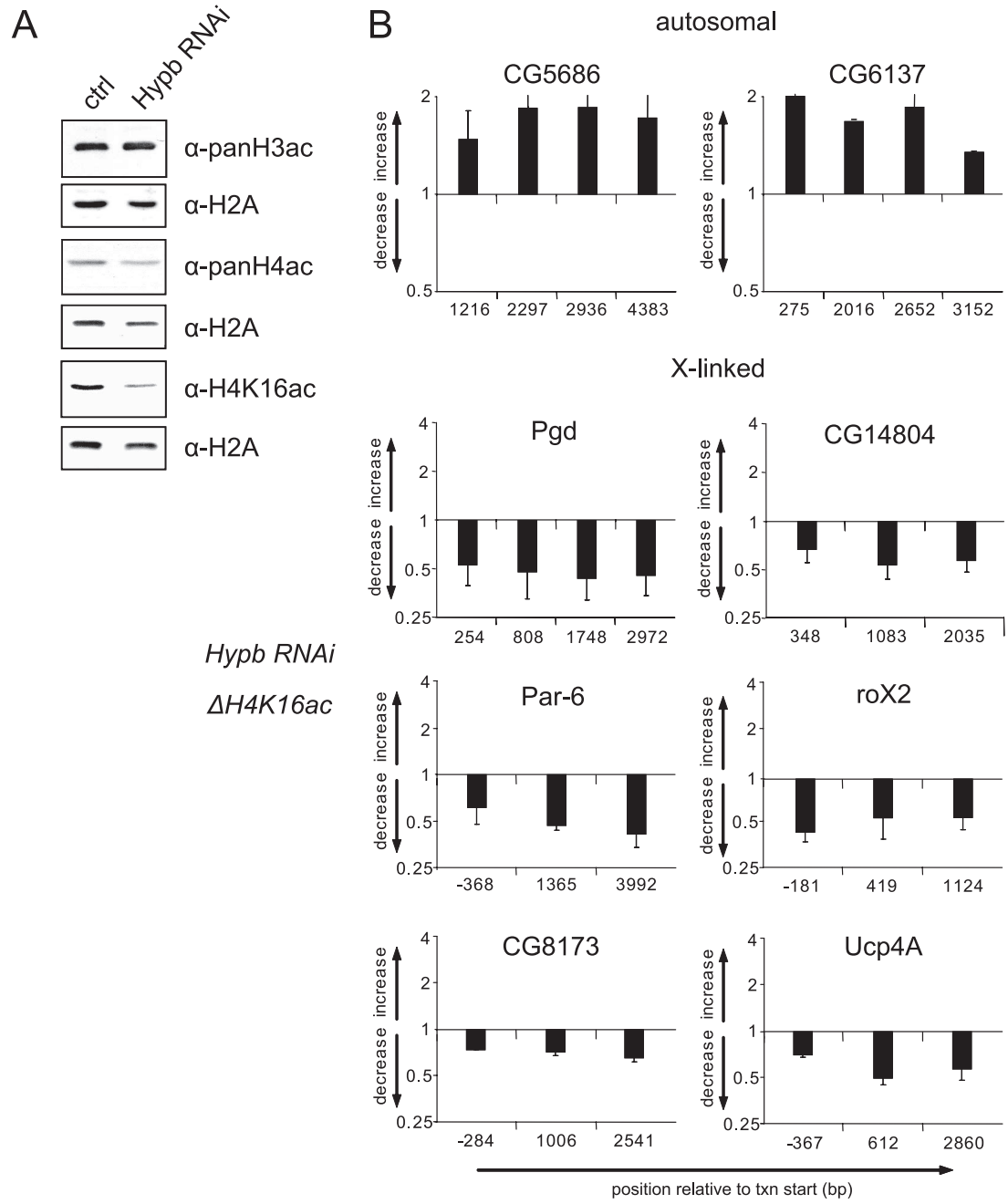


FIG. 4. H3K36 trimethylation is required for H4K16 hyperacetylation of the dosage-compensated X chromosome. (A) Hypb RNAi results in a reduction of bulk H4K16ac in male SL2 cells as indicated by Western blot analysis. (B) Comparison of changes in H4K16ac along autosomal and dosage-compensated X-linked genes upon RNAi by ChIP and real-time PCR. At autosomal genes, H4K16ac levels increase upon reduction of Hypb, whereas levels at dosage-compensated genes decrease.

binding and H4K16ac at the *roX2* gene under these conditions (Fig. 4B).

Hence, our results suggest that H3K36me3 is an important signal for stable association of a fully functional MSL complex with high-affinity sites and crucial for the binding of MSL proteins to low-affinity target genes. In the absence of trimethylation, MSL binding is diminished, resulting in lower levels of H4K16 hyperacetylation.

K36 methylation is required for transcriptional regulation at MSL target genes. Acetylation of H4K16 can relieve chromatin-mediated repression of transcription in vitro (1) and thus directly contribute to transcriptional upregulation of dosage-compensated genes. To address whether H3K36-dependent changes in acetylation affect transcription at target genes, we measured mRNA expression in untreated and Hypb knockdown cells by reverse transcription and quantitative real-time

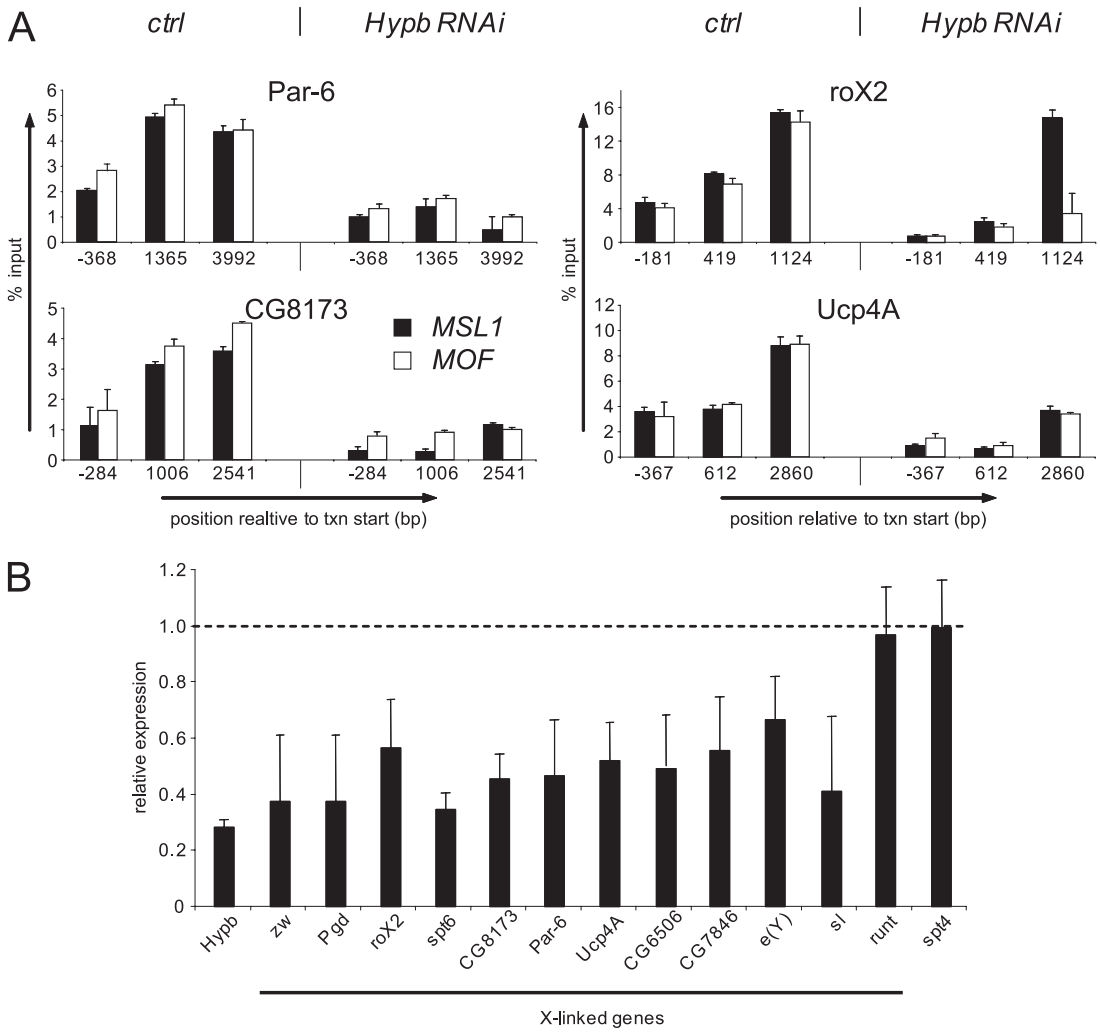


FIG. 5. H3K36 trimethylation enhances binding of the MSL complex and transcriptional upregulation at dosage-compensated genes. (A) MOF and MSL1 enrichments at dosage-compensated genes were compared with untreated and Hypb RNAi cells by ChIP and real-time PCR analysis. In control cells, both proteins displayed a similar pattern, localizing preferentially in the 3′ end of genes. Hypb knockdown led to diminished levels of MOF and MSL1 at all positions along *Par-6*, *CG8173*, and *Ucp4A*. MOF was also depleted from high-affinity sites along the *roX2* gene, while the level of MSL1 remained largely unaffected. (B) Relative mRNA expression from dosage-compensated and noncompensated genes after Hypb knockdown. Displayed are average mRNA levels of five independent experiments, normalized to a mitochondrial RNA, comparing untreated and Hypb knockdown cells by quantitative real-time PCR (14). Upon Hypb RNAi, mRNA expression levels of 11 dosage-compensated genes are reduced approximately twofold. Expression levels of noncompensated X-linked *runt* as well as autosomal *spt4* remain unchanged.

PCR. Indeed, expression of all tested dosage-compensated genes was significantly reduced upon decline of H3K36me3 (Fig. 5B). This effect was similar to the approximately twofold decrease of target gene mRNA levels after MSL2 knockdown (26), indicating that trimethylation is important for adequate transcriptional upregulation of X-linked genes. However, expression was not similarly decreased at all genes tested, since an X-linked gene, which is not subject to dosage compensation, and an autosomal gene remained unaffected. These results emphasize the critical role of H3K36me3 as a chromatin signature to allow recruitment of MSL proteins to sites of transcriptional compensation.

DISCUSSION

In this study, we report that trimethylation of histone H3 lysine 36 is required for high levels of H4K16ac at dosage-compensated genes on the male X chromosome. This function does not reflect an X-specific methylation signature, since both H3K36 methylation states have similar localization patterns at autosomal genes: dimethylation peaks promoter-proximal, and trimethylation shows a 3′ bias (Fig. 1). Furthermore, the regulation of H3K36me3 depends on the activity of Hypb, which is equally targeted to autosomal and X-linked loci, indicating a common mode of regulation (Fig. 2 and 3).

Nevertheless, downregulation of H3K36me3 in *Drosophila* SL2 cells resulted in reduced levels of H4K16 hyperacetylation at X-linked genes but simultaneously increased levels at autosomal genes in the same cells (Fig. 4). This differential effect on acetylation suggests a context-dependent readout of lysine 36 methylation. In *Saccharomyces cerevisiae*, H3K36me signals binding of the chromodomain-containing protein Eaf3, which in turn recruits an Rpd3 complex to deacetylate the 3′ end of

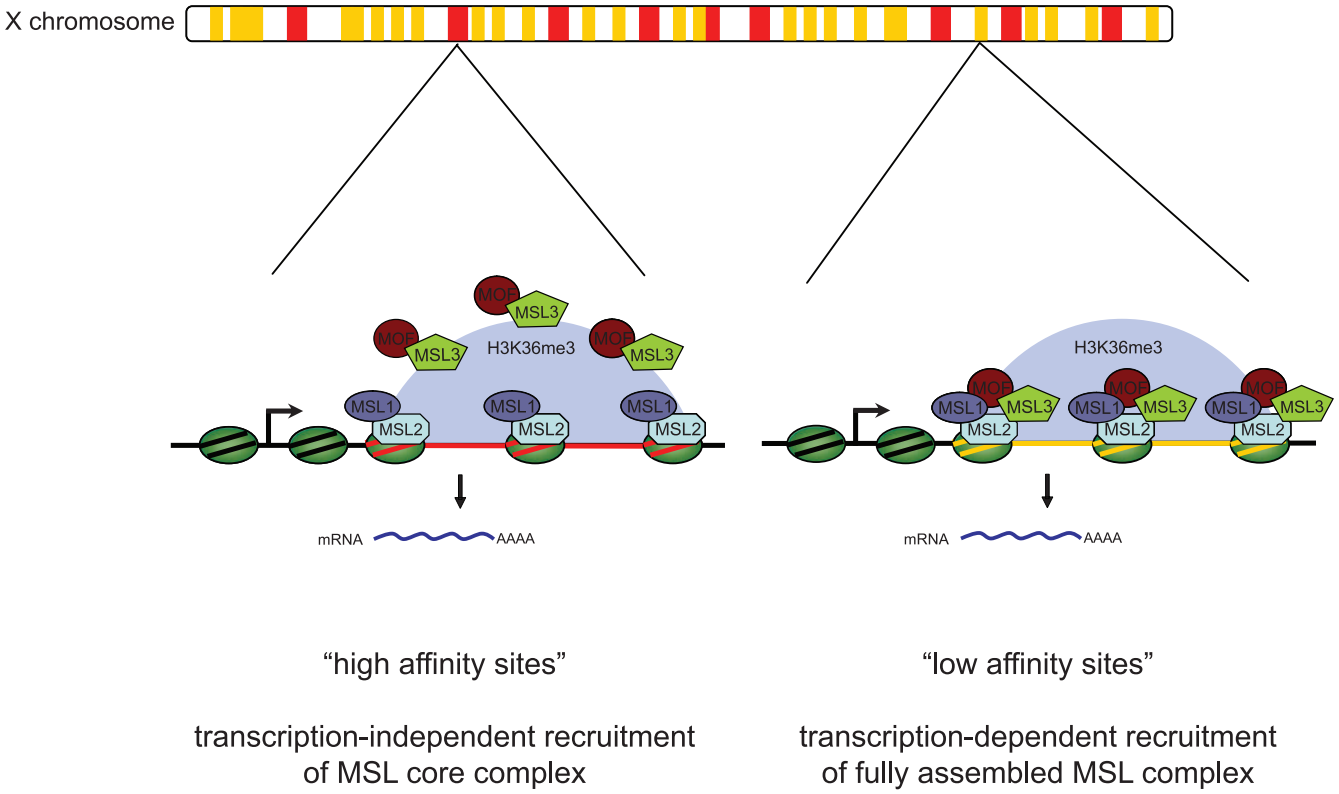


FIG. 6. Model for MSL complex targeting to sites of dosage compensation in *Drosophila*. Genes along the X chromosome have various affinities for MSL complex binding. High-affinity sites (red) can attract partially assembled MSL complexes independent of transcription-coupled chromatin modifications. However, H3K36me3 is still necessary to facilitate robust interaction with MOF and MSL3. In comparison, the majority of target genes contain promoter-distal sequence elements, which have relatively weak affinity for MSL recruitment (low-affinity sites [yellow]). At these genes, transcription-dependent H3K36me3 (blue arch represents concentration of H3K36me3) enhances recognition and stable binding of fully assembled MSL complexes. Thus, robust recruitment of the MSL proteins to the dosage-compensated X chromosome relies on combined contributions of degenerate sequence elements and transcription-coupled histone modifications.

transcribed genes (5, 11, 13). We provide evidence that the X-specific reduction of histone acetylation in Hypb-depleted *Drosophila* SL2 cells reflects compromised recruitment of MSL1 and MOF at dosage-compensated genes (Fig. 5A). This is in full agreement with reduced binding of MSL3 upon Hypb knockdown, which was recently reported by Larschan and colleagues (15). MSL3 is one of the *Drosophila* homologues of yeast Eaf3 (7) and localizes together with MOF and MSL1 to the 3′ end of dosage-compensated genes (2, 9). Thus, in analogy to yeast, MSL3 is likely to associate with H3K36me3 at the 3′ end of X-linked genes, leading to robust complex binding and enhanced H4K16 hyperacetylation. This is supported by evidence showing that MSL3 preferentially interacts with Set2-methylated nucleosomes in vitro (15). Moreover, our observation of Hypb localizing to active sites on polytene chromosomes provides further evidence for a direct role of H3K36me3 in MSL recruitment (Fig. 2B). However, not all sites enriched for Hypb were also bound by MSL1, suggesting that H3K36me3 is necessary but not sufficient for MSL complex recruitment.

Whereas proper binding of the MSL complex to *Par-6*, *CG8173*, and *Ucp4A* relies on the presence of H3K36me3, Hypb knockdown did not significantly decrease MSL1 recruitment at the *roX2* gene (Fig. 5A) (15). This is similar to the binding of MSL1 and MSL2 to high-affinity sites in *msl3* or *mof* mutant flies (10, 12, 17), suggesting that strong sequence affinity can target partial MSL complexes independent of H3K36me3. Importantly, despite its presence at the *roX2* locus in Hypb knockdown cells, MSL1 was insufficient for adequate MOF recruitment and transcriptional upregulation (Fig. 5A and B). Thus, our data indicate that H3K36me3 is necessary at high-affinity sites to facilitate robust MOF interaction and the subsequent hyperacetylation needed to double transcription (Fig. 6).

Interestingly, *roX2* transcription was unaffected by Hypb RNAi when expressed from a plasmid model system (29). Since the consequence of reduced H3K36me3 on H4K16ac on the *roX2* plasmid was not determined in this study, it is possible that a less pronounced reduction in acetylation might account for this effect.

In contrast to the *roX2* gene, H3K36me3 was required for MSL1 binding to lower-affinity genes. At these genes, transcription-dependent methylation might facilitate DNA accessibility in the 3′ end by enhancing the recruitment of MOF and the hyperacetylation of H4K16 (Fig. 6).

At autosomal genes, reduced trimethylation caused the opposite effect on H4 lysine 16 acetylation. Thus, one modification may signal two different outcomes in the same cell in a

chromosome-specific fashion. It is conceivable that such differential readouts involve interaction with either distinct methyl-binding proteins or alternative subunit compositions.

The presence of antagonistic activities in the same nucleus, which are targeted to the same modification, requires spatial restriction of individual protein complexes to avoid deregulation by improper acetylation or deacetylation. Thus, the preferential interaction of MSL proteins with H3K36me3 on the X chromosome might be favored by locally accumulating MSL proteins at high-affinity sites. MSL interactions with nuclear pore proteins (19) suggest a possible role of nuclear organization in X chromosome dosage compensation, which may further contribute to a preferential binding of MSL proteins to H3K36me3. Conversely, while this confines histone acetyltransferase activity to dosage-compensated genes on the X chromosome, it might also ensure that the same activity is not mistargeted to autosomal genes.

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